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Characteristics of the Nisin-Resistant Transformants of *Lactococcus lactis* subsp. *lactis* LM0230

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To investigate the nature and location of the nisin-resistance determinant of *Lactococcus lactis* subsp. *lactis* 7962 (*L. lactis* 7962), a total plasmid DNA prepared from *L. lactis* 7962, a nisin producer, was used to transform *L. lactis* subsp. lactis LM0230, a plasmid-free and nisin-sensitive strain, by protoplast mediated transformation procedures. All of the nisin-resistant transformants acquired the ability to utilize sucrose at the same time, confirming the close linkage between these two determinants in *L. lactis* 7962. The plasmid DNA profiles of a few selected nisin-resistant transformants were examined by agarose gel electrophoresis. No common plasmid was found among the transformants and some small plasmids previously not present in *L. lactis* 7962 were detected. These transformants were named as *L. lactis* KL1, KL2, KL3, KL4, or KL5, respectively based on their plasmid profiles. Growth curves of all transformants were similar to that of *L. lactis* LM0230, but different from that of *L. lactis* 7962. *L. lactis* KL5 showed the highest level of resistance to nisin, growing up to 1,200 IU nisin/ml after 40 hr incubation. Some nisin-sensitive derivatives of KL1 or KL2 were obtained by plasmid curing experiments. The plasmid DNA profiles of the nisin-sensitive KL1 derivatives were apparently the same as that of the KL1. All of the nisin-sensitive KL2 derivatives were plasmid-free, but a nisin-resistant strain with no apparent plasmid was also obtained. These results indicate that the nisin-resistance of the Nis^r transformants is presumably mediated by the chromosomally located gene(s) rather than plasmid-encoded gene(s).

Lactic acid bacteria produce a wide range of antagonistic substances including metabolic end-products such as lactic acid, antibiotic-like substances, and antimicrobial proteins or bacteriocins (18). Among the antimicrobial proteins produced by lactic acid bacteria, nisin has been studied most extensively so far (14, 19, 25).

Nisin is produced by certain strains of *L. lactis* subsp. *lactis*. It has a molecular weight of 3,500 Da and is composed of 34 amino acids, including 2,3-unsaturated amino acids and thioether amino acids (10). Nisin belongs to a group of bacteriocins termed "lantibiotics" (26). Lantibiotics are peptide-derived antibiotics with high antimicrobial activity against several pathogenic and spoilage Gram-positive bacteria, such as *Listeria*, *Staphylococcus*, and *Clostridium* (2, 14). They can be divided into two subgroups, i.e., linear shaped or globular lantibiotics. (17). They are first synthesized as precursor peptides, which are enzymatically processed to mature forms later (16). The unsaturated amino acids are responsible for the functionally important properties in the nisin molecule, i.e., thermostability and bactericidal action (19). They play a direct role by acting as electrophilic Michael acceptors toward nucleophiles in the cytoplasmic membrane. Nisin is currently used as a food preservative in some countries since it is able to inhibit the outgrowth of spores of *Bacillus and Clostridium* (3), the major flora responsible for canned food spoilage.

Several investigators have studied the genetics of lactococci in relation to both nisin production and resistance, and have attempted molecular cloning of involved genes. So far, conflicting results on the location of nisin -producing and resistance genes have been reported. Some researchers found that these genes were on the chromosome (4, 28) while others got evidences which indicated they were plasmid-encoded genes (8, 15). Re-

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cently, it has been shown that the structural gene for the nisin precursor (*spa*N) is located on chromosomal DNA (4, 5, 24). Moreover, *spa*N seems to form an operon with other genes for posttranslational processing within a novel conjugative transposon, Tn5301 (12). Tn 5031 is about 70-Kbp in size, carries sucrose fermenting gene(s), and is located on the chromosome. However, attempts to isolate a single plasmid which carries genes for sucrose fermentation and nisin production and resistance have not been successful. But a 64-Kbp plasmid, pNP40, responsible for both lactose fermentation and nisin resistance had been successfully isolated (21). Froseth *et al.* (7) reported that a 7.6-Kbp *Eco*RI fragment in pNP40, which also contained the origin of replication (*ori*), was responsible for nisin-resistance. However, pNP 40 was isolated from a strain which does not produce nisin and the 7.6 Kbp nisin-resistance determinant did not hybridize with the total genomic DNA from *L. lactis* ATCC11454, a nisin-producing strain. Therefore, the nature of the nisin-resistance conferred by pNP40 is unclear and it may be a completely different mechanism from that of *L. lactis* ATCC11454. Recently, von Wright *et al.* (32) cloned the nisin resistance gene from *L. lactis* 10.084, a nisin producer. It was located on the 46-Kbp plasmid, pSF01, which was associated with nisin-resistance, lactose fermentation, and proteinase activty but not with nisin-production or sucrose fermentation. Nisin-resistance gene can be used as a selection marker for food-grade cloning vectors in which all sequences should be originated from microorganisms approved for food use.

L. lactis ATCC7962 has been known as a strain with high activity of nisin production and resistance to nisin (22), but not much is known about the location of nisin-producing and resistance genes. Some conflicting results were reported (11, 29). According to Tsai and Sandine (29), a 28-Kbp plasmid, pNS17.5, seemed to be responsible for nisin-production and resistance in 7962 since when *Leuconostoc dextranicum* 181 acquired apparently pNS17.5 by conjugation, it became nisin producing and resistant strain. But more direct evidences were not presented. On the contrary, Harris *et al.* (11) reported that a probe containing pronisin (*spa*N) gene cloned from *L. lactis* ATCC11454 hybridized to a fragment generated by *Hind*III digestion of ATCC7962.

In this study, nisin-resistant transformants of *L. lactis* LM0230, a nisin-sensitive strain, were first obtained by transforming LM0230 cells with total plasmid preparations from *L. lactis* 7962, and then the characteristics of a few transformants were studied to investigate the nature and location of the nisin-production and resistance genes in *L. lactis* 7962.

MATERIALS AND METHODS

Strains, Media, and Culture Conditions

Strains used in this work are listed in Table 1. Stock cultures were stored at −20℃ in M17 medium (30) containing 25% glycerol. *L. lactis* 7962, a nisin producing strain, is able to ferment both lactose and sucrose and harbors six cryptic plasmids (29). *L. lactis* LM0230, a plasmid-free derivative of C_2 (6), was used as a host strain in the transformation experiments. M17 medium was used to culture *L. lactis* strains for analyzing growth, nisin-resistance, and preparing total genomic and plasmid DNA. All of the strains were grown at 30℃ without aeration using a 1% inoculum into an appropriate volume of M17 medium. Nisin-resistant transformants were grown in M17-G broth containing purified nisin (Aplin and Barrett, Trowbridge, England) at the concentration of 200 IU/ml (IU; international unit, approximate activity of 1 g of pure nisin is 40 IU).

Preparation of Plasmid DNA

Rapid small-scale and large-scale plasmid DNA preparations were done according to the methods described by Anderson and McKay (1). To make cells sensitive to lysozyme treatment, D,L-threonine was added to the final concentration of 20 mM to M17 broth. When required, plasmid DNA was further purified by cesium chloride-ethidium bromide equilibrium density gradients ultracentrifugations (20). Purified plasmids were examined by agarose gel electrophoresis. The plasmid profiles of *L. lactis* 7962 and transformants were analyzed by aga-

Table 1. Bacterial strains used in this work

Strain	Relevant phenotype	Comment (reference)	Plasmid profile (MDa)
L. lactis ssp. lactis			
ATCC7962	Lac^+, Suc^+, Nip^+, Nis^r	Parent (31)	45, 30, 18.5, 17.5, 14.5, 13
LM0230	Lac^-, Suc^-, Nip^-, Nis^s	plasmid-cured derivative of C_2 (6)	
KL1	Lac^-, Suc^+, Nis^r	This work	45, 30
KL2	Lac^+, Suc^+, Nis^r	This work	45, 30, 10, 6, 4, 3
KL3	Lac^+, Suc^+, Nis^r	This work	10, 6, 4, 3
KL4	Lac^+, Suc^+, Nis^r	This work	16, 13, 7
KL5	Lac^-, Suc^+, Nis^r	This work	35, 25
KL6	Lac^-, Suc^+, Nis^r	Nisin resistant strain cured from KL2	
KL7	Lac^-, Suc^-, Nis^r	Nisin sensitive strain cured from KL2	

Abbreviations: Lac^+; lactose utilizing, Suc^+; sucrose utilizing, Nip^+; nisin producing, Nis^r; nisin resistant, Nis^s; nisin sensitive.

rose gel electrophoresis using 0.8% agarose gel.

Protoplast-mediated Transformation

Protoplast-mediated transformation of *L. lactis* LM 0230 was done according to the method described by von Wright *et al.* (31), with some modifications. Protoplasting of the recipient strain, LM0230, was done by the method of Gasson (9), except for the lysozyme treatment which was done for 2 hr at 37℃ in SM17-G medium (M17-G medium containg 0.5 M sucrose as the osmotic stabilizer).

Selection of the Nisin-resistant Transformants

Nisin stock solution (20mg/ml) was prepared in 0.02 N-HCl as described by McKay and Baldwin (21), and diluted to the desired concentration in M17-G broth or M17-G agar (agar 1.5%) plate. The colonies growing on a M17-G agar plate supplemented with 200 IU nisin/ml both in top and bottom agar were selected as the nisin-resistant transformants. The plates were incubated at 30℃ for five to seven days before the transformants were counted. The transformants began to appear after 24~36 hr.

Physiological Characteristics of the Transformants

Growth curves: The growth curves of the few selected transformants were determined by measuring the absorbance of culture at 600 nm during growth. Fifty ml of fresh M17-G broth was inoculated with 0.5 ml of an overnight culture and incubated at 30℃. Aliquots were taken at 1 hr interval, diluted with distilled water if necessary, and the absorbance was measured. The growth curves of *L. lactis* 7962 and *L. lactis* LM0230 were also obtained for comparison.

Nisin-resistance: The degree of nisin-resistance of the transformants was compared with that of *L. lactis* 7962 by measuring the cell growth in M17-G medium containing 0, 200, 400, 800, 1600 IU of nisin per ml, respectively. The cell growth was determined by measuring the absorbance of culture at 600 nm after 20 and 40 hr of incubation.

Lactose and sucrose fermenting ability: Lactose or sucrose fermenting ability of transformants was tested by streaking the transformants on a M17-Lactose or M 17-Sucrose agar with bromocresol purple (40 mg/l) as a pH indicator; the amount of β-disodiumglycerophosphate was reduced to 5 g/l to lower the buffering capacity of M17 medium to a level where acid-producing colonies could be detected colorimetrically (27).

Plasmid-curing of the Transformants

Plasmid-curing of the transformants was carried out to examine whether nisin-resistance phenotype of the transformants is due to the acquisition of a specific plasmid from *L. lactis*. Curing was first tried by culturing the transformants at an elevated temperature (37℃) successively (27). Five ml of fresh M17 broth was inoculated with a specific transformant and the culture was incubated at 37℃ until it reached late log phase, and then diluted (1 : 100) with a fresh medium and reincubated at the same temperature until the late log phase was reached again. After seven cycles of consecutive subculturing, a portion of the culture was serially diluted to an appropriate concentration and then an aliquot was plated to give well isolated single colonies. The isolated colonies were tested for nisin-resistance by toothpicking on M17-G agar and M17-G agar containing 200 IU of nisin per ml. Nisin-sensitive colonies were selected and a plasmid DNA was prepared to examine their plasmid profiles.

RESULTS AND DISCUSSION

Plasmid Profiles of the Nisin-resistant Transformants

Total plasmid DNA from *L. lactis* 7962 was isolated and used to transform *L lactis* LM0230. The cells growing on a SM17-G agar plate supplemented with 200 IU of nisin per ml in both top and bottom agar were selected the nisin-resistant transformants. The transformants were appeared 24~36 hr later and the transformation efficiency was as low as 5 transformants/μg plasmid for the protoplast-mediated transformation. Plasmid DNA from the Nis^r transformants was isolated and visualized by agarose gel electrophoresis. When plasmid DNA profiles of the transformants were compared with that of the strain 7962 (Fig. 1), no common plasmid could be located among the Nis^r transformants and some small plasmids that were not present in *L. lactis* 7962 were detected in some transformants. The results could be interpreted as the presence of recombination phenomena or plasmid-linked restriction-modification systems in LM0230. Representative transformants were named as *L. lactis* KL1, KL2, KL3, KL4, and KL5 respectively according to the differences in plasmid profiles. All the nisin-resistant transformants were capable of fermenting sucrose confirming the close linkage between nisin-resistance and sucrose fermenting genes. Two of them (KL1 and KL5) were not able to utilize lactose thus showing Lac phenotypes. Although KL2 had two additional plasmids (about 30 and 45 kbp) when compared with KL3, both had the same phenotypes, Nis^r, Suc^+, and Lac^+. Therefore KL3 was excluded in later experiments.

Growth Curves of the Transformants

Growth curves of the Nis^r transformants were obtained as described in Materials and Methods section and are shown in Fig. 2. The exponential growth phase of the

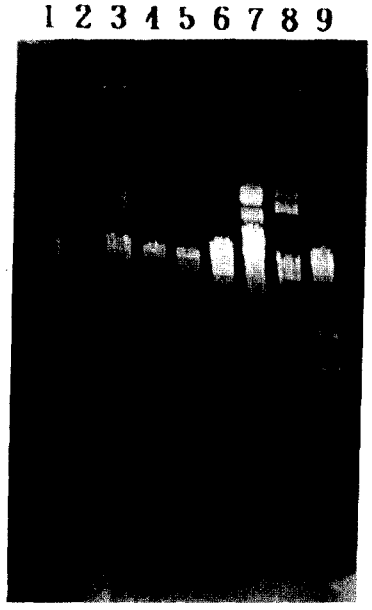

Fig. 1 Plasmid DNA profiles of the selected Nis^r transformants.

Total plasmid DNA preparation from *L. lactis 7962 was used to transform L. lactis* LM0230. Nis^r transformants were selected on M17-G agar plate containing 200 IU nisin/ml. Plasmid DNA from the transformants was isolated and visualized by agarose gel(0.8%) electrophoresis.

Lane 1, 9: *Hind*III-digested λ DNA(size marker), Lane 2: *L. lactis* LM0230, Lane 3: *L. lactis* KL1, Lane 4: *L. lactis* KL2 Lane 5: *L. lactis* KL3 Lane 6: *L. lactis* KL4 Lane 7: *L. lactis* KL5 Lane 8: *L. lactis* 7962.

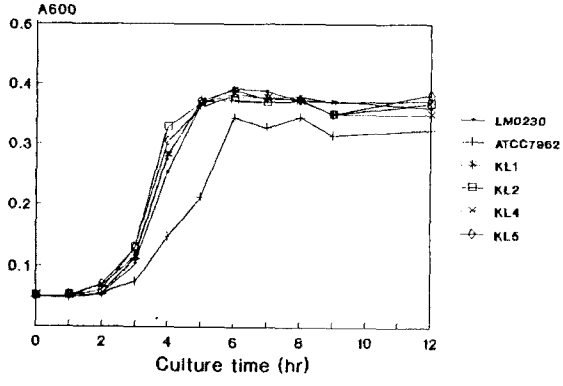

Fig. 2. Growth curves of the Nis^r transformants.

Transformants were grown in M17-G at 1% inoculum. Growth was monitored by measuring absorbance at 600 nm.

transformants took place between 3 and 5 hr after the onset of incubation. Growth curves of all Nis^r transformants examined were identical to that of LM0230, the host strain for transformation, but different from that of 7962. This result indicates that the growth pattern of LM0230 in M17-G media was not interfered by the introduction of the foreign plasmid DNA. Generation time for the transformants and LM0230 was approximately 0.8 hr but extended to 1.6 hr for 7962 under the same experimental conditions.

Degree of Nisin-resistance of the Transformants

The degree of nisin-resistance of the transformants was compared with that of 7962, a nisin producer, by measuring the cell growth in M17-G broth containing various concentrations of nisin. The cell growth was monitered by measuring the absorbance of the cell culture at 600 nm. Fig. 3 shows the absorbance of the culture,

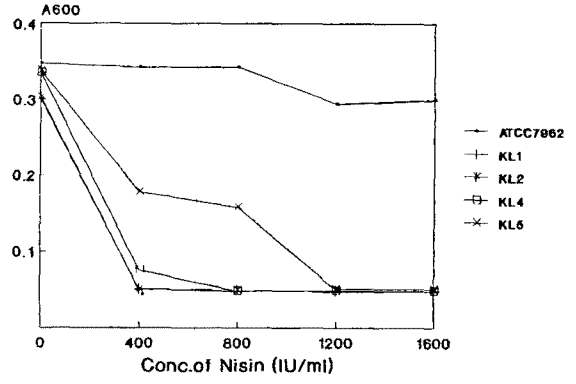

Fig. 3. Comparison of nisin resistance of the Nis^r transformants after 20 hr incubation.

Transformants were grown in M17-G broth containing 0, 200, 400, 800, and 1600 IU of nisin per ml, respectively. Degree of nisin-resistance was determined by measuring absorbance of the culture at 600 nm.

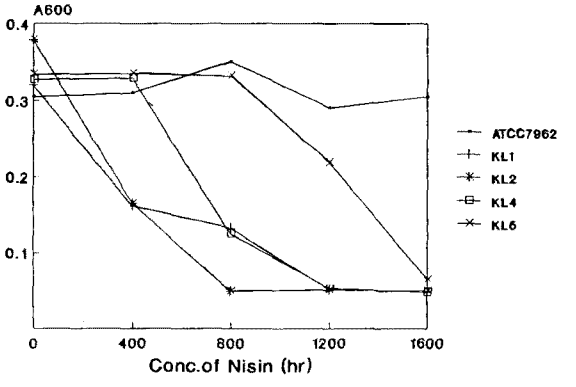

Fig. 4. Comparison of nisin resistance of the Nis^r transformants after 40 hr incubation.

Transformants were grown in M17-G broth containing 0, 200, 400, 800, and 1600 IU of nisin per ml, respectively. Degree of nisin-resistance was determined by measuring absorbance of the culture at 600 nm.

in other words, the degree of the nisin-resistance of the transformants at various nisin concentrations after 20 hr-incubation. Among the transformants, KL5 exhibited the highest level of resistance but the resistance level did not reach that of 7962. For example, *L. lactis* 7962 can grow in the presence of 1,600 IU nisin/ml without any significant reduction in the growth rate, but KL5 can not grow at 800 IU nisin/ml during 20 hr incubation period (1,600 IU nisin/ml in case of 40 hr incubation shown in Fig. 4). KL1 and KL2 exhibited the lowest nisin-resistance. They could not grow at 200 IU nisin/ml during the first 20 hr incubation period. Between 20 and 40 hr incubation period, some nisin-resistant cells began to appear among KL1 and KL2 populations. But KL1 cannot grow at 800 IU nisin/ml and KL2 cannot grow at 400 IU nisin/ml nor during 40 hr incubation period (Fig. 4). KL4 showed an intermediate degree of nisin-resistance. None of the transformants except for KL5 grew at 800 IU nisin/ml. KL5 showed the highest resistance to nisin among the nisin-resistant transformants. For *L. lactis* LM0230, it did not grow at 20 IU nisin/ml. It is not certain why different transformants showed difference in terms of nisin-resistance.

Plasmid Profiles of the Cured Transformants

Plasmid curing of the nisin resistant transformants was tried to examine whether changes in the plasmid profiles of transformants can directly cause any changes in the nisin-resistance phenotype. After curing at an elevated temperature of 37℃, as described in the Materials and Methods section, well isolated colonies were inoculated on a M17-G agar plate containing 200 IU nisin/ml with toothpick and screening of nisin sensitive strains were followed. Some colonies originating from KL1 and KL2 showed sensitivity to nisin but the colonies from KL4 and KL5 did not show any sensitivity to nisin. The plasmid DNA profiles of the nisin-sensitive strains derived from KL1 were apparently the same as that of the KL1 (results not shown). The relevant phenotypes of these strains were Nis^s and Suc^-. This result can be interpreted as that the nisin-resistance in KL1 could be mediated by chromosomally located gene(s).

Fig. 5 shows the screening result of the nisin-sensitive and resistant derivatives from the KL2, and Fig. 6, the plasmid profiles of the corresponding nisin-sensitive and resistant strains. The nisin-sensitive strains derived from KL2 were all plasmid-free, Lac , and Suc (Fig. 6 , lane 3 and 4). However, a plasmid-free strain (Fig. 6 , lane 2) with resistance to nisin was obtained (Fig. 5 , D), This strain also retained the abilities to utilize lactose and sucrose. These results again suggest that nisin-resistance gene(s) of KL2 may locate on the chromosome. This can be explained by the integration of nisin-resist-

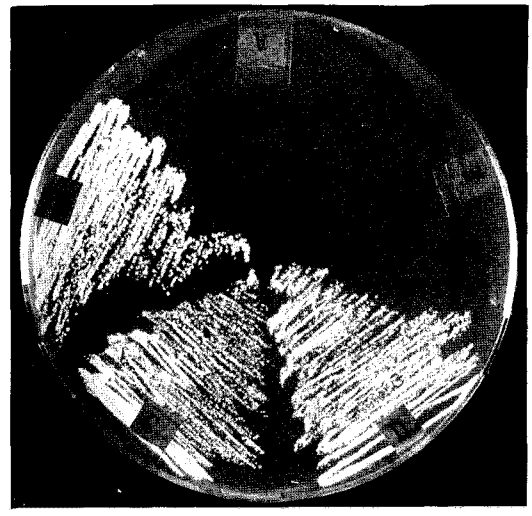

Fig. 5. Nisin-sensitive and nisin-resistant strains derived from *L. lactis* KL2 by successive culturing at 37°C.
Cured strains were screened by toothpicking on M17-G agar plate and M17-G agar plate containing 200 IU nisin/m.
A: *L. lactis* LM0230, B: *L. lactis* KL2, C, D: *L. lactis* KL6, nisin resistant strain cured form *L. lactis* KL2, E: *L. lactis* KL7, nisin sensitive strain cured from *L. lactis* KL2.

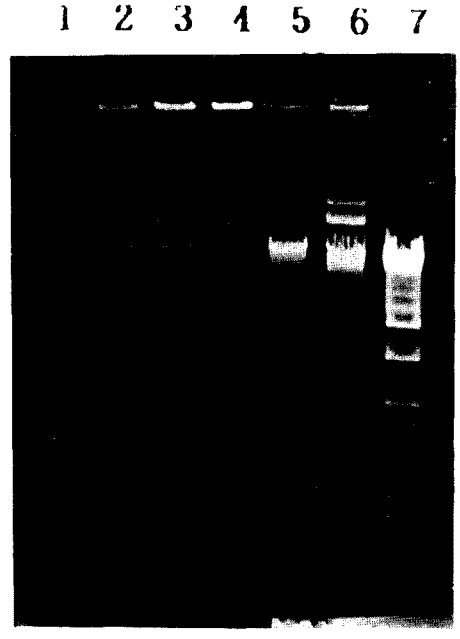

Fig. 6. Plasmid profiles of the selected strains obtained by curing of *L. lactis* KL2.
Plasmid DNA was isolated by the method of Anderson and McKay (1) and analyzed by electrophoresis in 0.8 % agarose gel at 8 V/vm.
Lane 1: *L. lactis* LM0230, Lane 2: Nisin resistant strain, Lane 3, 4: Nisin sensitive strains, Lane 5: *L. lactis* KL2 Lane 6: *L. lactis* 7962, Lane 7: *Hind*III-digested λ DNA (size marker).

ance gene together with sucrose or lactose utilization genes from a transposon present on the chromosome of *L. lactis* 7962 into the chromosome of *L. lactis* LM 0230 (12). During the total plasmid DNA preparation, this DNA segment somehow moved to a plasmid and then subsequently moved the LM0230 chromosome during transformation procedures. Another possibility is that the nisin-resistant plasmid of 7962 was unstable in LM0230 host background and that in some cases, the whole plasmid or part of plasmid incorporated into the LM0230 chromosome. Interqration of the lactose plasmid into the chromosome of *L. lactis* was reported by Petzel and McKay (23) and Hung *et al.* (13). Therefore the results from curing KL1 and KL2 suggest that nisin- resistance gene is probably mediated by chromosomal DNA.

CONCLUSION

The results obtained in the present study suggested that the nisin resistance of the Nis^r transformants is presumably mediated by the chromosomal DNA rather than the plasmid DNA. Growth curves of the Nis^r transformants were similar to that of *L. lactis* LM0230, the host strain of transformation, but different from that of *L. lactis* 7962. The degree of the nisin resistance of the transformants did not reach that of *L. lactis* 7962 which is a nisin producer. Among the transformants, *L. lactis* KL5 exhibited the highest resistance.

Futher studies would be necessary to clone the nisin-resistance determinant from *L. lactis* 7962 and use this gene as a food-grade selection marker in a replicon of the vector which originated from food-grade microorganisms.

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